Molecular Ecology Resources (2008) **8**, 1310–1312 doi: 10.1111/j.1755-0998.2008.02272.x

PERMANENT GENETIC RESOURCES

Development of simple sequence repeat markers for the soybean rust fungus, *Phakopsora pachyrhizi*

SHARON J. ANDERSON,* CHRISTINE L. STONE,* MARTHA LUCIA POSADA-BUITRAGO,†§ JEFFREY L. BOORE,†¶ BEENA A. NEELAM,‡** ROBERT M. STEPHENS,‡ DOUGLAS G. LUSTER,* REID D. FREDERICK* and KERRY F. PEDLEY*

**Foreign Disease-Weed Science Research Unit, Agricultural Research Service, US Department of Agriculture, Fort Detrick, Frederick, MD 21702, USA, †Joint Genome Institute and Lawrence Berkeley National Laboratory, US Department of Energy, Walnut Creek, CA 94598, USA, ‡Advanced Technology Program, Advanced Biomedical Computing Center, SAIC-Frederick Inc., NCI-Frederick, Frederick, MD 21702, USA*

Abstract

Twenty-four simple sequence repeat markers were developed for *Phakopsora pachyrhizi*, a fungal pathogen of soybean (*Glycine max*) and other legumes. All 24 of the loci were evaluated on 28 isolates of *P. pachyrhizi*. Twenty-one loci were polymorphic, with allelic diversity ranging from two to eight alleles, and null alleles were observed for eight of the 24 loci. A preliminary screen with the closely related species, *P. meibomiae*, indicated that these primer pairs are specific to *P. pachyrhizi*.

Keywords: basidiomycetes, microsatellites, *Phakopsora pachyrhizi*, soybean rust, SSR

Received 11 February 2008; revision accepted 29 March 2008

Soybean rust is an aggressive foliar disease caused by an obligate biotrophic fungus, *Phakopsora pachyrhizi*. The pathogen has spread globally, and the disease now occurs in all major soybean growing regions (Schneider *et al*. 2005). *P. pachyrhizi* reproduces asexually on soybean and all other known leguminous host plants through the production of dikaryotic urediniospores (Miles *et al*. 2003). The full life cycle of *P. pachyrhizi* has never been observed; thus, reproduction is predominantly, if not exclusively, asexual. Like other rust fungi, the prolific production of urediniospores, which can be dispersed thousands of kilometres by wind, contribute to the global distribution of the disease (Brown & Hovmoller 2002). Despite the negative impact caused by *P. pachyrhizi* on the worldwide production of soybeans, little is known about the genetic diversity of the pathogen. Here we present the development and characterization of 24 simple sequence repeat (SSR) markers for *P. pachyrhizi*.

SSR loci were identified bio-informatically from the *P. pachyrhizi* genomic sequence data available in GenBank using the programs RepeatMasker (Smit *et al*. (1996–2004) and STRFINDER (Shan & Stephens 2002). Twenty-four of the SSR loci (designated as PP001–PP024) identified were selected for further characterization. The efficacy of primers designed manually and using Primer3 software (Rozen & Skaletsky 2000) were initially evaluated using genomic DNA obtained from a single isolate of *P. pachyrhizi* as the template. Products generated with the SSR primers, ranging from 140 to 470 bp, were cloned and sequenced. BLAST searches revealed no significant similarity to other sequences.

The 24 unique SSR primer pairs were evaluated using 28 *P. pachyrhizi* isolates collected from 13 countries. Genomic DNA was isolated from urediniospores using the QIAGEN DNeasy Plant Mini Kit. All manipulations and propagation of the isolates were conducted inside the USDA-ARS, Foreign Disease–Weed Science Research Unit Biological Safety Level 3 (BSL-3) Plant Pathogen Containment Facility under the appropriate USDA-APHIS permit (Melching *et al*. 1983).

Correspondence: Kerry F. Pedley, Fax: (301) 619 2880;
E-mail: kerry.pedley@ars.usda.gov
Present addresses:
§Biophysics Laboratory, International Center of Physics, National University of Colombia, Ciudad Universitaria, Bogotá, Colombia.
¶Genome Project Solutions, 1024 Promenade Street, Hercules, CA 94547, USA.
**Monsanto Company, 700 Chesterfield Parkway West, St Louis, MO 63017, USA.

Journal compilation © 2008 Blackwell Publishing Ltd
No claim to original US government works

Table 1 Primer sequences, repeat motif, and PCR conditions for 21 *Phakopsora pachyrhizi* SSR loci

Locus	GenBank Accession no.	Primer sequence (5′–3′)	Repeat motif of cloned allele	T_a (°C)	Primer (nM)	No. of alleles (size range in bp)	H_E	H_O	P‡
PP002	EF193259	F: CCTCTGTCCCAAACATAAGC R: CTAGGTTCCAAGCTGTATTTC	$(AT)_{16}$	65	750	3 (200–223)	0.420	0.536	0.956
PP003	EF193260	F: GGCTCAGTCAAAGCATCCTC R: ATCAATTCTGGCCTGGTGAG	$(AT)_{15}$	65	500	4 (186–196)	0.635	0.071	0.000*
PP004	EF193261	F: ACTGTTCGGTTCGGTTTCAG R: CTTGGTTAAATGCCAAGCTTG	$(TC)_{13}$	65	500	4 (235–253)	0.524	0.679	0.330
PP006	EF193263	F: ACCCTGCCACTGGTAAGAGA R: TCCAAGACCCGTAGATGGTC	$(AGA)_{13}$	65	250	5 (159–179)	0.259	0.250	0.400
PP007	EF193264	F: GGTTGATGTTAAGATGGGTTGG R: CCTAGACTGTCAGGGTGAGAG	$(AG)_{15}$	68	250	2 (331–333)	0.233	0.038	0.002*
PP008	EF193265	F: GAACCTGTTCGGGCTGCTTTAGG R: GTAGCTTCTAATCTCAGGCGTC	$(AT)_9$	68	250	4 (198–207)	0.584	0.259	0.000*
PP009	EF193266	F: GGACTCTAGAACTGTGCACTAG R: GGTACTGTATGACCCTGCCTG	$(TTA)_{10}$	68	250	3 (336–342)	0.494	0.370	0.002*
PP010	EF193267	F: CTGAGTGAAATCACGCTGAGA R: GGCAGGTGATTCGTAGAGTCTAC	$(TGT)_{21}$	65	250	7 (205–281)	0.717	1.000	0.000*
PP011	EF193268	F: GACCCTTGTGACAGCTGATTG R: GAGTCAGCCTAAGCTCAGCCAC	$(TC)_{14}$	65	250	6 (242–256)	0.691	0.929	0.000*
PP012	EF193269	F: TGTAGCCCACCTTGAATCAG R: GTTGCCAAAAAGTGGGGTGAG	$(AT)_{12}$	65	250	2 (253–255)	0.198	0.074	0.030*
PP014	EF193271	F: CAGCGATCAGGTTCAGAAATC R: CCATCAGAGTTGTTGGCTCTC	$(CT)_{17}$	65	250	7 (279–293)	0.698	0.929	0.000*
PP015	EF193272	F: CAACCACGTGCACAACTATTC R: CCACCTCCTTTGAATCCTCA	$(TC)_{12}$	65	250	3 (462–471)	0.559	0.500	0.000*
PP016	EF193273	F: CAGGAAGACTCCAGAACTGTGC R: CCAAGGACACTTCTAGTCCTTC	$(CAAT)_{12}$ ... $(TTA)_{10}$	68	500	3 (320–348)	0.304	0.071	0.000*
PP017	EF193274	F: CGAGCCATTGCCCCAAGTTTG R: CAGTTAGATGAGCCTGAGGAC	$(TA)_{11}$	68	250	5 (208–216)	0.618	1.000	0.000*
PP018	EF193275	F: GGTCTGATCTAACAGGATCCAG R: GTTGTTGGAATCGGGTAGAGG	$(TGA)_{11}$	65	250	5 (124–144)	0.608	0.536	0.000*
PP019	EF193276	F: CCAAGTGCTGCAAATCAAGC R: GCTCTAACTAGAGCCCTTGTG	$(TA)_9$	68	500	3 (194–198)	0.575	0.444	0.022*
PP020	EF193277	F: CTTGAACAGAGTCATTATTTCTC R: CACCCTCATGTGCTTCTTAATTC	$(AAT)_{17}$	67	500	5 (239–257)	0.598	0.520	0.000*
PP021	EF193278	F: CAACGGCAAAAGACCTAGGTAC R: GCGCAGCCCTAACTACAATAC	$(TA)_{12}$	65	250	3 (335–338)	0.253	0.000	0.000*
PP022	EF193279	F: CAGCAGGTTAGTTCTGCACAGC R: TGGGTCATCAGAGCTTTCAG	$(TA)_{17}$	68	500	4 (157–163)	0.585	0.815	0.050
PP023	EF193280	F:CCATTCTAACAGTGATGAAGATAG R: GACAGAGGGTTGTGATCTTGG	$(TCA)_8$	67	500	8 (194–209)	†	†	
PP024	EF193281	F: CACTTCTCCTCCACAGCTGTAG R: CCAACATGTGATCATCCCCTC	$(AT)_6$	68	250	3 (139–145)	†	†	

F, forward primer; R, reverse primer; T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity.
*significant deviation from Hardy–Weinberg equilibrium.
†PP023 and PP024 did not behave in a diploid manner; thus, H_O and H_E were not calculated.
‡P values include Bonferroni correction.

Polymerase chain reaction (PCR) was performed in 12.5 μL reaction mixtures containing 1× Phusion buffer (New England Biolabs), 2.0 mM $MgCl_2$, 0.2 mM dNTP, 5 ng template DNA, and 0.1 U Phusion High-Fidelity DNA Polymerase (New England Biolabs) using a Gene AMP PCR System 9700 thermocycler (Applied Biosystems), with initial denaturation at 95 °C for 30 s, followed by 30–35 cycles of 94 °C for 30 s, 65–68 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. Primer sequences and concentrations and optimized annealing temperatures are listed in Table 1. The forward primers were labelled with FAM (6-carboxyfluorescein), and the resulting amplification products were

Journal compilation © 2008 Blackwell Publishing Ltd
No claim to original US government works

separated and detected by capillary electrophoresis on an ABI3130x1 (Applied Biosystems), using ROX500 as the internal size standard. Peak calls were made with ABI GeneMapper version 3.7 software.

The number of alleles per locus ranged from one to eight with an average of 3.9. Rust urediniospores are dikaryotic, and it was expected that the SSR markers would behave in a diploid manner, with a maximum of two products for each nonduplicated locus. Two of the 24 markers, PP023 and PP024 (Table 1), produced polyploid peak patterns that could not be resolved to diploid patterns in several of the isolates. Since duplication events and linkage among loci are unknown for the *P. pachyrhizi* genome, these markers were excluded from further analyses.

Among the remaining 22 SSR loci analysed, three (PP001, PP005, PP013) were invariant (with respect to product length) among the 28 *P. pachyrhizi* isolates screened. Of the remaining 19 loci, one had a single invariant allele (PP002). All other loci were polymorphic among the *P. pachyrhizi* isolates, ranging from two to seven alleles at each locus (Table 1).

Primers sets that produced no PCR product for a particular isolate were scored as a null allele when no amplicon was obtained in at least three separate polymerase chain reactions and the sample DNA and SSR primer pair both gave positive results in control experiments using different primer/DNA combinations. Eight loci (PP007, PP008, PP009, PP012, PP019, PP020, PP022, and PP023) contained null alleles among the 28 isolates tested, and were restricted to seven loci in a single isolate from Vietnam, and two loci in two Hawaiian isolates.

The average heterozygosity across the entire collection of *P. pachyrhizi* isolates was 0.41, deviating from Hardy–Weinberg equilibrium (HWE). Excluding the invariant loci, heterozygosity ranged from a low of 0.038 to a high of 1. Sixteen of the loci exhibited significant deviation from HWE, using an exact test based on 2000 switches and corrected for multiple comparisons (Rousset 2008). The majority of these loci (50%) had levels of heterozygosity lower than expected. Linkage disequilibrium was calculated for pairwise combinations of loci using the program GenePop (Rousset 2008). Only invariant loci (PP001, PP005, and PP013) were excluded from the analysis. Ninety-two per cent of the pairwise combinations showed significant linkage disequilibrium ($P \leq 0.05$). Given that the isolates used in this study were from asexual populations of *P. pachyrhizi*, these results are not surprising.

Two *P. meibomiae* isolates were used to test for cross-species amplification with the 24 primer pairs. Only primers for two of the markers, PP012 and PP016, yielded any detectable product when *P. meibomiae* genomic DNA was used as the template. However, the amount of product was negligible compared to the amount produced when *P. pachyrhizi* genomic DNA was used as the template. The results indicate that either the microsatellite loci are not present in the *P. meibomiae* genome and/or that the flanking DNA in these regions is not well conserved.

Acknowledgements

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the US Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

References

Brown JK, Hovmoller MS (2002) Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science*, **297**, 537–541.

Melching JS, Bromfield KR, Kingsolver CH (1983) The plant pathogen containment facility at Frederick, Maryland. *Plant Disease*, **67**, 717–722.

Miles MR, Frederick RD, Hartman GL (2003) Is the US soybean crop at risk? APSnet feature, June 2003. *American Phytopathological Society*, St Paul, Minnesota. Online publication. http://www.apsnet.org/online/feature/rust/.

Rousset F (2008) GenePop'007: a complete re-implementation of the GenePop software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103–106.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

Schneider RW, Hollier CW, Whitam HK *et al*. (2005) First report of soybean rust caused by *Phakopsora pachyrhizi* in the continental United States. *Plant Disease*, **89**, 774.

Shan J, Stephens RM (2002) *StrFinder: A Perfect Tandem Repeat Finding Program*. SAIC-Frederick, NCI-Frederick. http://www-fbsc.ncifcrf.gov/app/htdocs/appdb/appinfo.php?appname=StrFinder.

Smit AFA, Hubley R, Green P (1996–2004) *RepeatMasker Open-3.0*. http://www.repeatmasker.org.

Journal compilation © 2008 Blackwell Publishing Ltd
No claim to original US government works